

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|   |           |  |
|---|-----------|--|
| <b>(51) International Patent Classification <sup>6</sup>:</b><br><b>C07K 16/28, C12N 5/20, A61K 39/395,</b><br><b>G01N 33/577</b>   | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 95/09873</b><br><b>(43) International Publication Date:</b> 13 April 1995 (13.04.95)   |
| <b>(21) International Application Number:</b> PCT/US94/11305<br><b>(22) International Filing Date:</b> 6 October 1994 (06.10.94)<br><b>(30) Priority Data:</b><br>08/132,619 6 October 1993 (06.10.93) US<br><b>(71) Applicant:</b> BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).<br><b>(72) Inventor:</b> VITETTA, Ellen, S.; 6914 Pemberton Drive, Dallas, TX 75230 (US).<br><b>(74) Agent:</b> WILSON, Mark, B.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).  |           | <b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> A MONOCLONAL ANTI-HUMAN IL-6 RECEPTOR ANTIBODY<br><br><b>(57) Abstract</b><br><br>This application relates to antibodies directed against the IL-6 receptor and hybridomas capable of producing such antibodies. In some embodiments of the invention, these antibodies are used to inhibit the binding of IL-6 to an IL-6 receptor. In other embodiments, the antibodies are used to inhibit the proliferation of IL-6 dependent cells. Finally, a kit for the inhibition of IL-6 dependent proliferation is disclosed. |           |  |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                  | GB | United Kingdom                        | MR | Mauritania               |
| AU | Australia                | GE | Georgia                               | MW | Malawi                   |
| BB | Barbados                 | GN | Guinea                                | NE | Niger                    |
| BE | Belgium                  | GR | Greece                                | NL | Netherlands              |
| BF | Burkina Faso             | HU | Hungary                               | NO | Norway                   |
| BG | Bulgaria                 | IE | Ireland                               | NZ | New Zealand              |
| BJ | Benin                    | IT | Italy                                 | PL | Poland                   |
| BR | Brazil                   | JP | Japan                                 | PT | Portugal                 |
| BY | Belarus                  | KE | Kenya                                 | RO | Romania                  |
| CA | Canada                   | KG | Kyrgyzstan                            | RU | Russian Federation       |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan                    |
| CG | Congo                    | KR | Republic of Korea                     | SE | Sweden                   |
| CH | Switzerland              | KZ | Kazakhstan                            | SI | Slovenia                 |
| CI | Côte d'Ivoire            | LI | Liechtenstein                         | SK | Slovakia                 |
| CM | Cameroon                 | LK | Sri Lanka                             | SN | Senegal                  |
| CN | China                    | LU | Luxembourg                            | TD | Chad                     |
| CS | Czechoslovakia           | LV | Latvia                                | TG | Togo                     |
| CZ | Czech Republic           | MC | Monaco                                | TJ | Tajikistan               |
| DE | Germany                  | MD | Republic of Moldova                   | TT | Trinidad and Tobago      |
| DK | Denmark                  | MG | Madagascar                            | UA | Ukraine                  |
| ES | Spain                    | ML | Mali                                  | US | United States of America |
| FI | Finland                  | MN | Mongolia                              | UZ | Uzbekistan               |
| FR | France                   |    |                                       | VN | Viet Nam                 |
| GA | Gabon                    |    |                                       |    |                          |

- 1 -

DESCRIPTIONA MONOCLONAL ANTI-HUMAN IL-6 RECEPTOR ANTIBODY

5

BACKGROUND OF THE INVENTIONField of the Invention

10

This invention generally relates to cytokines and the role which they play in the immune response. More specifically, the invention relates to antibodies directed against IL-6 and methods of using such

15 antibodies.

Background of the Related Art

Interleukin-6 (IL-6) is a highly pleiotropic

20 cytokine which plays a critical role in the immune response, in acute phase reactions and in hematopoiesis (1-5). It is produced by T and B cells, macrophages, bone marrow stromal cells, fibroblasts, macrophages, and endothelial cells (1). Previous reports suggest that

25 deregulated IL-6 production may be involved in the pathogenesis of many diseases, including multiple myeloma, rheumatoid arthritis, mesangial proliferative glomerulonephritis, Castleman's disease and Kaposi's sarcoma (2). In this regard, IL-6 is a major growth

30 factor for myeloma cells and can participate in both autocrine and paracrine growth (3,4). A recent clinical trial has demonstrated that the treatment of patients with monoclonal anti-IL-6 antibodies (mAbs) can suppress the proliferation of multiple myeloma cells *in vivo* and

35 completely inhibit the bioactivity of IL-6 (5).

- 2 -

The IL-6 receptor (IL-6R) has two functionally different chains; a 80 kDa IL-6-binding protein (IL-6R), and a 130kDa signal-transducing protein (gp130). Gp130 does not bind to IL-6, but it is involved in the formation of high affinity IL-6-binding sites (6). Monoclonal anti-IL-6R antibodies which neutralize the bioactivity of IL-6 may facilitate the study of the structure-function relationship of the IL-6R and may be good candidates for therapeutic reagents in the treatment of IL-6-related diseases such as multiple myeloma (7,8).

Several mAbs with similar characteristics have been described by other investigators (18,19). Novick and colleagues (18) have prepared a panel of mAbs by immunization of mice with purified soluble hIL-6R from human urine. Some of these mAbs inhibited the binding of labeled sIL-6R to IL-6 in a solid phase RIA. These antibodies have been used for affinity purification and detection of the soluble hIL-6R. Hirata and coworkers (19) have also described two mAbs against the hIL-6R. One inhibited the binding of IL-6 to U266 cells and blocked IL-6-dependent growth of a human T cell lymphoma cell line *in vitro*. Recently, Suzuki, et al. (8) have demonstrated that the administration of one of the anti-IL-6R antibodies to SCID mice with human myeloma xenografts, strongly inhibited tumor growth. Similar experiments with UV4 are in progress. In this regard Sata, et al. (20) have humanized their anti-IL-6R antibody as a prelude to using it in humans.

30

IL-6 plays a central role in the pathogenesis of multiple myeloma and the growth of myeloma cells. Hence, patients with myeloma cells that are responsive to IL-6 might benefit from therapy with anti-IL-6R-based therapy.

- 3 -

SUMMARY OF THE INVENTION

A monoclonal antibody (UV4) against the human IL-6 receptor (hIL-6R) was generated by immunizing BALB/c mice with both a human myeloma cell line (U266) and a murine cell line (M12.4/R) transfected with the hIL-6R cDNA. Flow cytometric analysis demonstrated that UV 4 stains the hIL-6R<sup>+</sup> cell lines U266 and U937, but not the hIL-6R<sup>-</sup> cell lines Daudi and K562. Competitive inhibition assays demonstrated that preincubation of U266 cells with UV4 inhibited the binding of a phycoerythrin (PE)-IL-6 conjugate to the hIL-6R and also inhibited the proliferative activity of IL-6 on the IL-6-dependent human myeloma cell lines LKM2 and ILKM3. In contrast, UV4 did not interfere with the proliferation of the hIL-6R<sup>-</sup> Burkitt's lymphoma cell line, Daudi. Direct sandwich radioimmunoassays further confirmed that the UV4 bound to the same molecule as the goat anti-hIL-6R antibody. These results suggest that both UV4 and human IL-6 bind to the same or adjacent epitopes on the hIL-6R. This monoclonal antibody should facilitate studies of the structure-function relationship of IL-6R and may be useful for the treatment of IL-6-dependent diseases such as multiple myeloma.

Because of the technical difficulty in obtaining large amounts of purified hIL-6R protein, the inventors expressed the hIL-6R on a murine cell line instead of purifying the hIL-6R protein for immunization. To this end, the inventors cloned a truncated hIL-6R gene lacking the cytoplasmic domain from human myeloma U266 cells into eukaryotic expression vector, then transfected the recombinant vector into the IL-6R<sup>-</sup> murine cell line, M12.4.

Flow cytometric analysis indicates the antibody (UV4) specifically reacts with hIL-6R<sup>+</sup> cell lines U266

- 4 -

and U937, but not with hIL-6R<sup>+</sup> cell lines, Daudi and K562. In the competitive inhibition assay, UV4 efficiently blocked the binding of IL-6-PE conjugate to its receptor on U266 cells. Furthermore, UV4 inhibited the IL-6-induced proliferation of human bone marrow-derived myeloma cell lines ILKM-2 and ILKM-3, but had no effect on the Burkitt's lymphoma cell line, Daudi or on the IL-6-dependent murine cell lines, as shown in (3H)-thymidine incorporation assays. A direct sandwich RIA, further confirmed that UV4 recognized the same molecule as GAsIL-6R. UV4 could sterically interfere with IL-6 binding by binding an epitope adjacent to the IL-6 binding site or to the IL-6 binding-site itself.

The present invention deals generally with anti-IL-6 receptor monoclonal antibodies. Such antibodies have the ability to bind to the IL-6 receptor. In preferred embodiments, the antibodies of the invention are able to detectably bind to cells that express the IL-6 receptor on their surface. Examples of such cells include U226, U937, and M12.4/R cell lines. The binding of the anti-IL-6 receptor antibodies allows for the presence of IL-6 receptors to be diagnosed by such methods as, for example, flow cytometric analysis, sandwich assays, and <sup>3</sup>H-Thymidine incorporation assays.

Preferred monoclonal antibodies of the invention are capable of inhibiting the binding of IL-6 to an IL-6 receptor. Further, the antibodies of the invention are capable of interfering with proliferative activity of certain IL-6 dependent cells. For example, it has been shown that such antibodies can inhibit the proliferative activity of IL-6 on myeloma cell lines such as, ILKM-2 and ILKM-3 in a dose-dependent manner. A particularly preferred embodiment of the present invention is the UV4 monoclonal antibody, the production and characteristics

- 5 -

of which are detailed in the Detailed Description of the Preferred Embodiment Section of this application.

In other embodiments, the invention involves  
5 hybridomas capable of expressing the antibodies of the invention. For example, hybridomas expressing the UV4 antibody are a preferred embodiment of this aspect of the invention.

10 Methods of inhibiting the binding of IL-6 molecules to IL-6 receptors are also included in this invention. These methods involve obtaining antibodies that are made according to the invention, then binding the antibodies to an IL-6 receptor in a manner that inhibits the binding  
15 of IL-6 molecules to the receptor. This inhibition can be of IL-6 receptors of intact cells, or on IL-6 receptors which have been more or less purified from cells.

20 The inhibition of binding of IL-6 to the IL-6 receptor can be further used in methods of inhibiting the proliferative activity of IL-6-dependent cells. Such methods are further embodiments of the invention. These methods involve: obtaining an anti-IL-6 receptor  
25 antibody; treating IL-6-dependent cells that are susceptible to inhibition by the anti-IL-6 receptor antibody with the antibody; binding the antibody to an IL-6 receptor; and thereby inhibiting the proliferative activity of the cell. In more preferred embodiments, the  
30 proliferative activity of IL-6-dependent myeloma cells can be inhibited by these methods. For example, proliferation of IL-6-dependent human myeloma cell lines such as ILKM-2 and ILKM-3 can be inhibited in this manner. Further, proliferation of myeloma cells *in vivo*  
35 may be inhibited in this manner.

- 6 -

The ability of anti-IL-6 receptor antibodies to inhibit the proliferation of IL-6-dependent cells can be employed to enhance a population of cells that is not susceptible to inhibition by anti-IL-6 receptor antibodies relative to a population of cells that is susceptible to such inhibition. For example, this method can be used to enhance a population of Daudi cells relative to a population of ILKM-2 cells in cell culture. Further, the method can be used to enhance the population of non-myeloma cells relative to a population of myeloma cells. This enhancement of one population of cells relative to another can occur in either *in vivo* or *in vitro* situations.

Other embodiments of the present invention involve kits for inhibiting the proliferative activity of an IL-6-dependent cell. Such kits will comprise an anti-IL-6 receptor antibody. Such a kit may also include a method for introducing the antibody into the location in which inhibition of the IL-6-dependent cells is desired.

Other embodiments of the present invention include methods of detecting an IL-6 receptor. These methods include the steps of: obtaining an anti-IL-6 receptor antibody; treating a sample suspected of having an IL-6 receptor with that antibody; and determining whether the sample binds to an IL-6 receptor in the sample. The IL-6 receptor to be detected can be in either whole cells, or in a more or less purified form. For example, the sample could be a cell lysate, a soluble protein portion of a cell, a membrane fraction of a cell, or in a more purified form of the receptor protein. Various detection methods can be employed, for example, sandwich radioimmunoassays and ELISA assays.



- 7 -

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Amplification of hIL-6R cDNA by PCR. lane  
a: DNA markers ( $\kappa$  DNA digested with ECOR 1 and Hind  
5 111); lane b, lane c: PCR products from PCR reaction  
with 1% formamide (lane b) or without formamide (lane c).

FIG. 2. Construction of the hIL-6R expression  
vector pCMV5/R.

10

FIG. 2A. Truncated hIL-6R gene.

FIG. 2B. Recombinant plasmid pCMV5/R. S, signal  
sequence; E, extracellular domain; T, transmembrane  
15 domain; I, intracellular domain; hGH, human growth  
hormone transcription terminator; CMV, human  
cytomegalovirus promotor; AMP<sup>r</sup>, Ampicillin resistance  
gene.

FIG. 3A, FIG. 3B, FIG. 3C and FIG. 3D show flow  
cytometric analysis of the UV4 staining on various human  
cell lines: (FIG. 3A) U266, myeloma cell line; (FIG. 3B)  
20 U937, histiocytic lymphoma cell line; (FIG. 3C) Daudi,  
Burkitt's lymphoma cell line; and (FIG. 3D) K562,  
25 erythromyeloid leukemia cell line.

FIG. 4A and FIG. 4B show that UV4 inhibits the  
binding of PE-IL-6 conjugate to IL-6R: (FIG. 4A) Control  
antibody MOPC-21; and (FIG. 4B) UV4.

30

FIG. 5A, FIG. 5B and FIG. 5C show that UV4 inhibits  
the proliferation of human myeloma cells: (FIG. 5A) ILKM2  
cells; (FIG. 5B) ILKM3 cells; and (FIG. 5C) Daudi cells.  
O-control antibody MOPC-21; ●-UV4.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTSMaterials and Methods5 Cell lines and cell culture:

The hIL-6R<sup>+</sup> cell lines U266 and U937 and the hIL-6R<sup>-</sup> cell lines Daudi and K562 were obtained from the American Type Culture Collection (Rockville, MD). The mouse IL-  
10 6R<sup>-</sup> cell line, M12.4, was a gift from Dr. Philip Tucker (University of Texas Southwestern Medical Center, Dallas, TX). These cell lines were maintained in RPM1-1640 supplemented with 10% fetal calf serum (FCS), glutamine, penicillin and streptomycin. The IL-6-dependent human  
15 myeloma cell lines ILKM2 and ILKM3 (9) were kindly provided by Dr. Shiro Shimizu (Kanazawa Medical University, Ishikawa, Japan). The IL-6-dependent murine cell lines KD83 and 7TD1 were gifts from Dr. R. Coffman (DNAX, Palo Alto, CA) and Dr. A. Tong (Baylor University  
20 Medical Center, Dallas, TX) respectively. These IL-6-dependent cell lines were maintained in RPM1-1640 in the presence of 2ng/ml recombinant human IL-6 (rhIL-6, Promega, Madison, WI).

25 RNA extraction:

Total RNA was isolated from U266 cells by the guanidine isothiocyanate method described by Sambrook et al. (10). Briefly, approximately  $1 \times 10^8$  fresh U266  
30 cells were washed with sterile phosphate-buffered saline (PBS) and resuspended in 4 ml of GIT buffer (4M guanidine isothiocyanate, 0.1 M Tris-HCl pH 7.5, 0.1%  $\beta$ -mercaptoethanol). The cell lysate was homogenized with a tissue grinder on ice for one minute. The lysate was  
35 centrifuged through CsCl buffer (5.7M CsCl, 25mM sodium acetate, pH 6.0) at 32000 rpm in a SW55 rotor (Beckman, Palo Alto, CA) for 16 hours at 20°C. The RNA pellet was

- 9 -

resuspended in 300  $\mu$ l of 0.3M sodium acetate, pH6.0 and precipitated by the addition of 750  $\mu$ l cold ethanol and incubated at -70°C for one hour. The precipitated RNA was collected by centrifugation, washed with 80% cold ethanol, dried under vacuum and dissolved in DEPC (dimethyl pyrocarbonate, Sigma) -treated distilled water. The amount of RNA was determined spectrophotometrically.

### Example I

10

#### Expression of hIL-6R

##### Cloning and expression of hIL-6R gene:

15 Using reverse transcription and PCR, the inventors have constructed a truncated hIL-6R gene, which lacks the intracytoplasmic domain. Initial attempts to synthesize and amplify IL-6R cDNA from first-strand cDNA using standard PCR (16), were unsuccessful. Consequently, the  
20 inventors added 1% formamide to the PCR reaction mixture (17). FIG. 1 demonstrates that PCR products from the reaction with formamide yielded a band of IL-6R cDNA. The IL-6R gene was then inserted into the mammalian expression vector pCMV5. The resulting construct  
25 (pCMV5/R) contained a 25bp 5' untranslated region plus the full coding sequence of the extracellular and transmembrane domains and a small portion of the intracellular domain for a total insert size of 1500 bp (FIG. 1, FIG. 2).

30

The pCMV5/R construct was cotransfected into the mouse lymphoma cell line M12.4 along with the pSV2-Neo plasmid by electroporation. After the initial selection in neomycin (G418), IL-6R expression on the transfectants  
35 was detected by a radioactive binding assay. The expression of hIL-6R in the transfected cells as shown in Table 1.

- 10 -

TABLE 1

Detection of the hIL-6R expression on the  
transfected cell line by RIA

|   | Cell Line                                   | CPM  |
|---|---|------|
| 5 | U266  | 3141 |
|   | M12.4 <sup>a</sup>                          | 268  |
|   | M12.4/R <sup>b</sup> M12.4/R-2 <sup>c</sup> | 1915 |
|   | M12.4/R-3 <sup>c</sup>                      | 3383 |
|   | M12.4/R-7 <sup>b</sup>                      | 3867 |
|   |   | 4849 |

10

- a untransfected cell line  
b transfected cell line  
c subclones from the transfected  
cell line M12.4/R

15

As can be seen, the binding of IL-6 to transfected M12.4  
cells was much greater than its binding to untransfected  
cells (M12.4), suggesting that transfectants express hIL-  
20 6R on their surface and that this IL-6R specifically  
binds to hIL-6. The pool of resistant transfectants  
which expressed hIL-6R were subcloned by limiting  
dilution and the clones with the highest expression were  
chosen to immunize BALB/c mice.

25

**Construction of the hIL-6R expression vector:**

The oligodeoxynucleotide primers for synthesizing  
hIL-6R first-strand cDNA and PCR amplification were  
30 derived from the published sequences of the hIL-6R (11).  
The sequences of the primers are as follows: forward  
primer, 5'-TTATCTAGATAAGCTGGCATGGCAGT-3' (SEQ ID NO:1);  
reverse primer, 5'-ATTATCGATGGAGTGGTAGCCGAGGA-3' (SEQ ID  
NO:2). The primers span 1500 bp of the hIL-6R coding  
35 region (Nucleotide sequences -25 to 1481). E. Coli

- 11 -

strain NM 522 cells and eukaryotic expression plasmid vector PCMV5 were a gift from Dr. Yan-Ting Zhou (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas). The vector

5 PCMV5 (12) contains the human cytomegalovirus (CMV) promoter, the human growth hormone transcription (hGH) termination and polyadenylation signals, and the SV40 origin of replication and enhancer.

10 First strand hIL-6R cDNA was synthesized from U266 RNA using a first-strand cDNA synthesis kit (Pharmacia, Piscataway, NJ). The first-strand cDNA was further amplified by the polymerase chain reaction (PCR) using

15 Taq polymerase in the presence of 1% formamide (Gibco, Gaithersburg, MD) and a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The amplified cDNA was cloned into the polylinker region of pCMV5. The recombinant plasmid, designated pCMV5/R, was then used to transform bacteria, E Coli the NM522. Plasmid DNA was extracted from

20 transformed bacterial clones and the transformed clones which contained plasmid pCMV5/R were verified by restriction enzyme analysis. A large amount of plasmid pCMV5/R from the transformed clone was purified by equilibrium centrifugation in CsCl-ethidium bromide

25 gradients (13).

#### Transfection and expression of hIL-6R:

The IL-6R<sup>-</sup> murine B cell lymphoma cell line M12.4

30 was cotransfected with plasmids pCMV5/R and pSV2-Neo by electroporation (Biorad, Hercules, CA). Cells were grown in complete RPMI-1640 medium. After 48 hours, the cells were selected by the addition of 0.5 mg/ml G418 (Gibco, Gaithersburg, MD). The G418-resistant transfectants were

35 subcloned by limiting dilution and further analyzed for expression of the hIL-6R.

- 12 -

Detection of the cell surface hIL-6R:

The rhIL-6 was radioiodinated with  $\text{NaI}^{125}$  using the Iodogen reagent (Pierce, Rockville, IL) (14). The cells  
5 were incubated for one hour on ice with  $^{125}\text{I}$ -rhIL-6 in 2% bovine serum albumin (BSA) in (PBS) containing 0.1% sodium azide and then washed three times with PBS. The cell-bound radioactivity was determined on a gamma  
10 counter. The U266 and M12.4 cell lines served as positive and negative controls, respectively.

Example IIProduction of mAb UV415 Generation of the mAb anti-hIL-6R:

The mAb UV4 was generated by immunizing mice with the IL-6R<sup>+</sup> cell line, U266 and the transfected M12.4/R cells. Initial characterization of primary hybridoma  
20 cultures and cloned hybridomas was performed by testing the supernatants against the IL-6R<sup>+</sup> cell lines U266 and M12.4/R and the IL-6R<sup>-</sup> cell lines K562 and M12.4 by a cellular ELISA. A mAb UV4 which reacted with U266 and M12.4/R, but not with K562 and M12.4 was selected for  
25 further study. Isotyping analysis demonstrated that the UV4 was an IgG<sub>1</sub>k.

Production of monoclonal anti-hIL-6R antibody:

30 BALB/c mice were immunized twice at monthly intervals with  $5 \times 10^6$  U266 cells and then injected i.p. three times with M12.4/R cells. Three days before fusion, the mice were injected intravenously (i.v.) with  $1 \times 10^7$  M12.4/R cells. Splenocytes from immunized mice  
35 were fused with mouse myeloma cells SP2/0 using 40% polyethylene glycol (PEG) and grown in hypoxanthine/aminopterin/thymidine (HAT) medium (15).

- 13 -

Supernatants from growing clones were screened by a cellular ELISA for antibodies against U266, M12.4/R, K562 and M12.4. Hybridomas reacted with U266 and M12.4/R, but not K562 and M12.4. The specific monoclonal antibodies (mAbs) were further screened by flow cytometry.

The mAbs were purified from culture supernatants by affinity chromatography with Gamma-binding G-agarose column (Gene Corporation, Gaithersburg, MD).

10

#### Cellular ELISA:

The 96-well ELISA plates were coated for 30 minutes with 50  $\mu$ l poly-L-lysine (0.01% in PBS, Sigma, St. Louis, MO). After removing the poly-L-lysine,  $1 \times 10^5$  cells per well were centrifuged onto the plates at 2000 RPM for 10 minutes. The cells were fixed for 15 minutes with 0.5% glutaraldehyde, and the plates were washed three times with PBS. 50  $\mu$ l of 1% BSA in PBS containing 100mM glycine was added and incubated for 45 minutes. The plates were then blocked with 150  $\mu$ l per well of 2% BSA in PBS containing 0.05% sodium azide and stored at 4°C for use. After three washes with PBS, 50  $\mu$ l hybridoma supernatants were added to each well and the plates were incubated for 1 hour at room temperature. The plates were washed again and incubated with 50  $\mu$ l of diluted goat anti-mouse Ig-(GAMIg) -alkaline phosphatase conjugate (Tago, Burlingame, CA) for an additional hour. The plates were washed and developed for 30 minutes with phosphatase substrate paranitrophenylphosphate (Sigma). 50  $\mu$ l per well of 3 M NaOH were then added to stop the reaction. The absorbance of each well was determined at 405 nm in a ELISA reader (Nippon Intermed K.K., Tokyo, Japan).

35

- 14 -

Antibody isotyping:

An ELISA isotyping kit was used to determine the isotype of antibody secreted (Boeringer Mannheim  
5 Biochemicals, Indianapolis, IN).

Flow cytometric analysis of UV4:

Flow cytometric analysis was performed with UV4  
10 using the hIL-6R<sup>+</sup> cell lines U266 and U937 and the IL-6R<sup>-</sup>  
cell lines Daudi and K562. The histograms presented in  
FIG. 3 show that a majority of the cells from the U266  
and U937 lines were specifically stained with UV4. There  
was no detectable staining with UV4 on K562 and Daudi  
15 cells. The results indicate that UV4 binds to IL-6R<sup>+</sup>  
cells, but not to IL-6R<sup>-</sup> cells.

In order to determine the specificity of the mAb,  
10<sup>6</sup> cells were incubated for 30 minutes on ice with 1 µg  
20 of primary antibody or an isotype-matched irrelevant  
murine IgG1k protein MOPC-21 (Cappel, Durham, NC) in 100  
µl of staining buffer (PBS containing 2% BSA and 0.05%  
sodium azide). The cells were washed with cold staining  
buffer and then stained with fluorescent isothiocyanate-  
25 conjugated GAMIg (FITC-GAMIg, Sigma, St. Louis, MI). The  
cells were washed again and analyzed on the FACScan  
(Becton-Dickinson, CA). Positively stained cells were  
determined by subtracting values obtained with the  
negative control using the Lysis II computer for data  
30 analysis.

For the competitive inhibition assay, 1 X 10<sup>5</sup> U266  
cells were incubated for 1 hour on ice with various  
concentrations of the purified mAb (UV4) or an isotype-  
35 matched control, MOPC-21 in 50 µl of staining buffer.  
0.05 µg of PE-hIL-6 (R & D system, Minneapolis, MN) was  
added for an additional hour on ice. The cells were



- 15 -

washed three times to remove unbound PE-IL-6 and antibodies, and resuspended in 0.2 ml of PBS for flow cytometric analysis. The PE-Avidin conjugate (R & D system) served as the control for nonspecific binding.

5

### Example III

#### Use of UV4 to Detect UIL-6R

UV4 recognizes the same molecule as the goat anti-hIL-6R antibody:

10

A direct sandwich RIA (radioimmunoassay) was carried out to further confirm whether the antigen recognized by UV4 was the IL-6R. As shown in Table 2, UV4 recognizes the same molecule as GASIL-6R where the isotype-matched myeloma protein MOPC-21 and the control detecting antibody,  $^{125}\text{I}$ -GAOVA do not. This result demonstrates that the antigen captured by UV4 is the hIL-6R molecule.

20

**TABLE 2**

Detection of the hIL-6R in Cell Lysates by a Direct Sandwich Radioimmunoassay

|       | UV4<br>(CPM) | MOPC-21<br>(CPM) |
|-------|--------------|------------------|
| Daudi | 839*         | 346              |
| U266  | 35502        | 916              |
| PBS   | 1249         | 743              |

25

\*  $^{125}\text{I}$ -GAOVA subtracted

30

In the RIA, UV4 was the capturing antibody while  $^{125}\text{I}$ -goat anti-human soluble IL-6R antibody (GASIL-6R, R & D Systems) was used for detection. GASIL-6R was iodinated with  $\text{Na}^{125}\text{I}$  as described above. For preparation of cell lysates,  $1 \times 10^7$  cultured Daudi or

35

- 16 -

U266 cells were lysed in 1 ml of digitonin lysis buffer (1% digitonin, 10 mM triethanolamine, PH 7.8, 10 mM iodoacetamide, 0.15M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml of leupeptins, 1  $\mu$ g/ml of pepstatin A and 1  $\mu$ g/ml of antipain) on ice for 20 minutes, and centrifuged to remove cell debris. Ninety-six well RIA plates (Fisher, Plano, TX) were incubated with 50  $\mu$ l/well of 20  $\mu$ g/ml of UV4 or the control myeloma protein MOPC-21 in PBS overnight at 4° C. The plates were washed 3 times with 0.05% TWEEN-20 PBS (PBST) and incubated for an additional 2 hours at room temperature with 50  $\mu$ l/well (80,000 cpm) of  $^{125}$ I-GASIL-6R or  $^{125}$ I- goat anti-ovalbumin antibody (GAOVA) as an additional control. The plates were again washed and the radioactivity in each well was determined by a gamma counter. The nonspecific binding of  $^{125}$ I-GAOVA was served as background.

( $^3$ H)-thymidine incorporation assay:

Cells were distributed into wells of flat-bottomed 96-well plates (Costar, Cambridge, MA) at  $1 \times 10^4$  per well and incubated for 1 hour with a serial dilution of UV4, MOPC-21 or medium. 1 ng/well of hIL-6 was then added (for IL-6-dependent cell lines only). After 72 hours of culture, the cells were pulsed for 24 hours with 1  $\mu$ Ci/well of ( $^3$ H)-thymidine (Amersham, Arlington Heights, IL). The cells were harvested onto glass filter paper using an automated cell harvester (Skatron, Sterling, VA) and the incorporated radioactivity was measured by a liquid scintillation counter (LKB). The results were expressed as a percentage of the ( $^3$ H)-thymidine incorporated by cells treated with medium and rIL-6 only.

- 17 -

**REPLACEMENT SHEET**Example IVUse of UV4 to Bind to and Inhibit IL-6 ActivityEffect of mAb UV4 on the Binding of IL-6 to U266:

5

A competitive binding assay was used to determine whether the mAb UV4 blocks the binding of IL-6 to the hIL-6R. The results are shown in FIG. 4. Preincubation of U266 cells with UV4 inhibited the binding of a PE-IL-6 conjugate to U266 cells in a dose-dependent manner. However, preincubation with the isotype-matched control antibody, MOPC-21 did not affect the binding of a PE-IL-6 conjugate. These results demonstrate that both UV4 and PE-IL-6 bind to the same or adjacent epitopes on the hIL-6R.

15

Inhibition of the Biological Activity of IL-6 by UV4:

Having determined that the binding of IL-6 to the hIL-6R could be inhibited, the inventors further examined the effect of the UV4 on the proliferation of the IL-6-dependent human myeloma cell lines ILKM-2 and ILKM-3. The cells were cultured with various amounts of UV4 in the presence of a constant amount (2 ng/ml) of rhIL-6. The cells were then pulsed with (3H)-thymidine. FIG. 5 shows that UV4 inhibits the proliferative activity of IL-6 on the myeloma cell lines ILKM2 and ILKM3, in a dose-dependent manner. The IC<sub>50</sub> of UV4 on both cell lines was  $1.5 \times 10^{-8}$  M. In contrast, the irrelevant murine IgG1 MOPC-21 had no effect. UV4 also did not interfere with proliferation of IL-6R<sup>+</sup> human cell line, Daudi. Although the growth of murine cell lines 7TD1 and KD83 is dependent on hIL-6, UV4 did not affect the growth of either line (data not shown).

35

\* \* \*

- 18 -

While the above examples demonstrate various preferred embodiments of the present invention, they are in no way meant to be limiting upon the scope of the invention. The skilled artisan will recognize that many variations of the invention are possible without deviating from the nature and spirit of the claimed invention. All such possibilities are to be considered to be part of the invention.

- 19 -

## REFERENCES

The following references are hereby incorporated in  
5 pertinent part to the present application.

1. Hirano, T. Interleukin-6 (IL-6) and its receptor:  
their role in plasma cell neoplasias. Int. J. Cell.  
Cloning, 9:166-184, 1991.  
10
2. Kishimoto, T., Akira, S., and Taga, T. Interleukin-6  
and its receptor: A paradigm for cytokines. Science,  
258:593-596, 1992.
- 15 3. Kawano, M., Hirano, T., Matsuda, T., Taga, T.,  
Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O.,  
Tanaka, H., and et al., Autocrine generation and  
requirement of BSF-2/IL-6 for human multiple myelomas.  
Nature, 332:83-85, 1988.
- 20 4. Klein, B., Zhang, X.G., Jourdon, M., Content, J.,  
Houssian, F., Aarden, L.A., Piechaczyk, M., and Bataille,  
R. Paracrine rather than autocrine regulation of myeloma  
cell growth and differentiation by interleukin-6. Blood,  
25 73:517-526, 1989.
5. Klein, B., Wijdenes, J., Zhang, X.G., Jaurdan, M.,  
Boiron, J.M., Brochier, J., Liautard, J., Clement, M.M.,  
Morel-Fournier, B., Lu, Z.Y., Mannoni, P., Sany, J., and  
30 Bataille, R. Murine anti-interleukin-6 monoclonal  
antibody therapy for a patient with plasma cell leukemia.  
Blood, 78:1198-1204, 1991.
6. Hibi, M., Murakami, M., Saito, M., Hirano, T., and  
35 Taga, T. Molecular cloning of an IL-6 signal transducer,  
gp130. Cell, 63:1149-1157, 1990.

- 20 -

7. Burlogie, B. Pathophysiology of human multiple myeloma-recent advances and future directions. *Current Topics in Microbiology and Immunology*, 182:245-250, 1992.
- 5 8. Suzuki, H., Yasukawa, K., Saito, T., Goitsuka, R., Haegawa, A., Ohsugi, Y., Taga, T., and Kishimoto, T. Anti-human interleukin-6 receptor antibody inhibits human myeloma growth in vivo. *Eur J Immunol*, 22:1989-1993, 1992.
- 10 9. Shimizu, S., Yoshioka, R., Hirose, Y., Sugai, S., Tachibana, J., and Konda, S. Establishment of two interleukin-6 (B cell stimulatory factor/interferon-beta 2)-dependent human bone marrow-derived myeloma cell
- 15 lines. *J Exp Med*, 169:339-344, 1989.
10. Sambrook, J., Fritsh, E.F., and Maniatis, T. Extraction, purification and analysis of messenger RNA from eubaryotic cells. In: *Molecular Cloning, A*
- 20 *Laboratory Manual*, pp. 19-22, New York: Cold Spring Harbor Laboratory Press. 1989.
11. Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., and
- 25 Kishimoto, T. Cloning and expression of the human interleukin-6 (BSF-2/IFN-beta 2) receptor. *Science*, 241:825-828, 1988.
12. Anderson, S., Davis, D.N., Dahback, H., Jomvall, H.,
- 30 and Russell, D.W. Cloning, structure and expression of the mitochondrial cytochrome p-450 steroid 26-hydroxylase, a bile acid biosynthetic enzyme. *J Biol. Chem*, 264:8222-8229, 1989.
- 35 13. Sambrook, J., Fritsh, E.F., and Maniatis, T. *Plasmid vectors*. In: *Molecular cloning, a laboratory manual*, pp. 1-42, New York: Cold Spring Harbor Press. 1989.

- 21 -

14. Shen, G-L., Li, J-L., Ghetie, M-A., Ghetie, V., May, R.D., Till, M., Brown, A.N., Relf, M., Knowles, P., Uhr, J.W., Janossy, G., Amlot, P., Vitetta, E.S., and Thorpe, P.E. Evaluation of four CD22 antibodies as ricin A  
5 chain-containing immunotoxins for the *in vivo* therapy of human B-cell leukemias and lymphomas. *Int. J. Cancer*, 42:792-797, 1988.
15. Goding, J.W. Production of monoclonal antibodies.  
10 In: *Monoclonal Antibodies: Principles and Practices*, pp. 59-103, New York: Academic Press. 1983.
16. Sambrook, J., Fritsh, E.F., and Maniatis, T. In  
15 vitro amplification of DNA by the polymerase chain reaction. In: *Molecular cloning, a laboratory manual*, pp. 18-20, New York: Cold Spring Harbor Press. 1989.
17. Sarkar, G., Kapelner, S., and Sommer, S. Formamide  
20 can dramatically improve the specificity of PCR. *Nucleic Acids. Res*, 18:7465, 1990.
18. Novick, D., Engelmann, H., Revel, M., Leitner, O.,  
and Rubinstein, M. Monoclonal antibodies to the soluble human IL-6 receptor: affinity purification, ELISA, and  
25 inhibition of ligand binding. *Hybridoma*, 10:137-146, 1991.
19. Hirata, Y., Taga, T., Hibi, M., Nakano, N., Hirano, T., and Kishimoto, T. Characterization of IL-6 receptor  
30 expression by monoclonal and polyclonal antibodies. *J. Immunol.*, 143:2900-2906, 1989.
20. Sata, K., Tsuchiya, M., Saldanha, J., Koshihara, Y., Ohsugi, Y., Kishimoto, T., and Bending, M.M. Reshaping a  
35 human antibody to inhibit the interleukin-6-dependent tumor cell growth. *Cancer Res*, 53:851-856, 1993.

- 22 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

NAME: BOARD OF REGENTS, THE UNIVERSITY OF  
TEXAS SYSTEM

STREET: 201 West 7th Street

10

CITY: Austin

STATE: Texas

COUNTRY: United States of America

POSTAL CODE: 78701

TELEPHONE NO: (512)499-4462

15

TELEFAX: (512)499-4523

## (ii) INVENTORS: VITETTA, Ellen

(iii) TITLE OF INVENTION: A MONOCLONAL ANTI-HUMAN IL-6  
RECEPTOR ANTIBODY

20

## (iv) NUMBER OF SEQUENCES: 2

## (v) CORRESPONDENCE ADDRESS:

25

(A) ADDRESSEE: Arnold, White & Durkee

(B) STREET: P.O. Box 4433

(C) CITY: Houston

(D) STATE: Texas

(E) COUNTRY: USA

30

(F) ZIP: 77210

## (vi) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

35

(C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII

(D) SOFTWARE: Wordperfect 5.1



- 23 -

## (vii) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: Unknown
- (B) FILING DATE: Concurrently herewith
- (C) CLASSIFICATION: Unknown

5

## (viii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: USSN 08/132,619
- (B) FILING DATE: October 6, 1993

10

## (ix) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Parker, David L.
- (B) REGISTRATION NUMBER: 32,165
- (C) REFERENCE/DOCKET NUMBER: UTFD350P--

15

## (x) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 512-418-3000
- (B) TELEFAX: 713-789-2679
- (C) TELEX: 79-0924

20

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- 25 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

30

TTATCTAGAT AAGCTGGCAT GGCAGT

26

## (2) INFORMATION FOR SEQ ID NO:2:

35

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid

- 24 -

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

5

ATTATCGATG GAGTGGTAGC CGAGGA

26

- 25 -

CLAIMS

1. An anti-IL-6 receptor monoclonal antibody, said  
5 antibody being further defined as:

detectably staining U226 and U937 cells but not K562  
or Daudi cells in a flow cytometric analysis;

- 10 being capable of inhibiting the binding of IL-6 to  
an IL-6 receptor; and

being capable of interfering with a proliferative  
activity of an IL-6 dependent cell.

15

2. The antibody of claim 1, further defined as UV4.

- 20 3. A hybridoma capable of expressing an antibody  
according to claim 1.

4. A method of using an antibody according to claim 1  
25 to inhibit the binding of an IL-6 molecule to an IL-6  
receptor comprising:

obtaining an antibody according to claim 1;

- 30 binding the antibody to the IL-6 receptor; and

inhibiting the binding of the IL-6 molecule to the  
IL-6 receptor.

35

- 26 -

5. A method of using an antibody according to claim 1 to inhibit proliferative activity of an IL-6 dependent cell that is susceptible to inhibition by an anti-IL-6 receptor antibody, comprising:

5

obtaining an antibody according to claim 1;

treating an IL-6 dependent cell that is susceptible  
to inhibition by an anti-IL-6 receptor antibody  
with the antibody;

10

binding the antibody to an IL-6 receptor; and

inhibiting proliferative activity of the cell.

15

6. The method of claim 5, further defined as a method for enhancing a population of cells that are not susceptible to inhibition by an anti-IL-6 receptor antibody relative to a population of cells that are susceptible to inhibition by an anti-IL-6 receptor antibody.

20

7. The method of claim 6, further defined as a method for enhancing a population of cells that are not susceptible to inhibition by an anti-IL-6 receptor antibody relative to a population of cells that are susceptible to inhibition by an anti-IL-6 receptor antibody where both populations of cells are contained in a culture vessel.

25

30

8. The method of claim 6 where the population of cells susceptible to inhibition comprises myeloma cells.

35

- 27 -

9. A kit for inhibiting a proliferative activity of an IL-6 dependent cell that is susceptible to inhibition by an anti-IL-6 receptor antibody, comprising an antibody of claim 1.

5

10. A method of using an antibody of claim 1 to detect an IL-6 receptor, comprising:

- 10        obtaining an antibody according to claim 1;
- treating a sample suspected of having an IL-6  
                                 receptor with the antibody; and
- 15        determining whether the antibody binds to an IL-6  
                                 receptor in the sample.

1/7

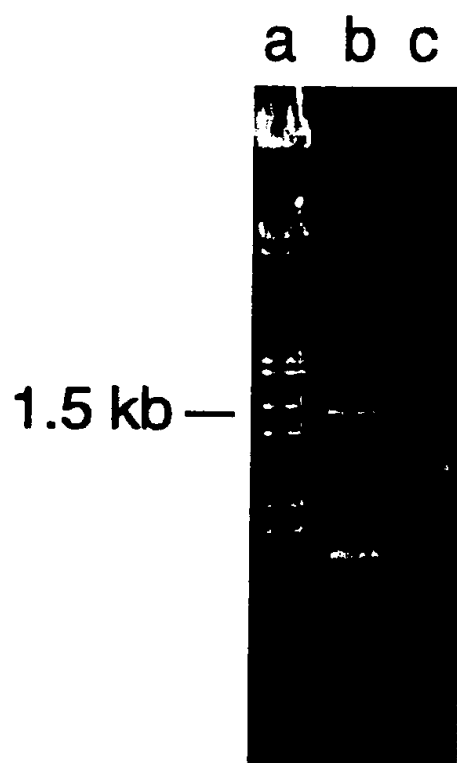


FIG.1

2/7

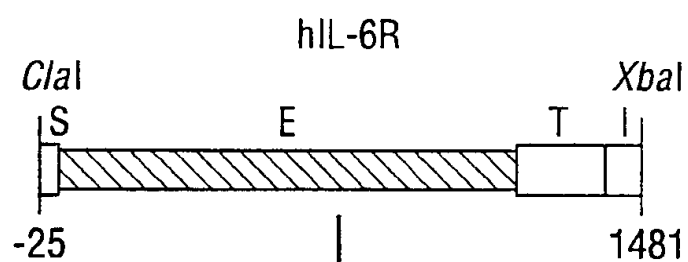


FIG. 2A

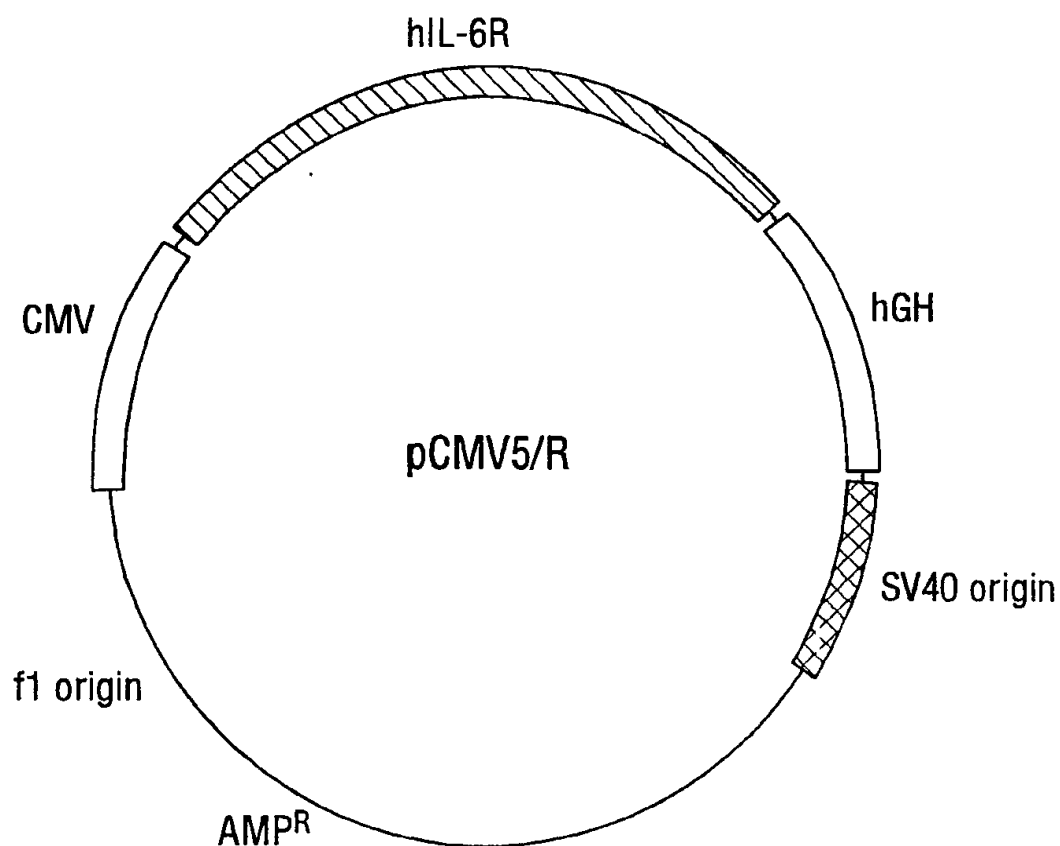


FIG. 2B

3/7

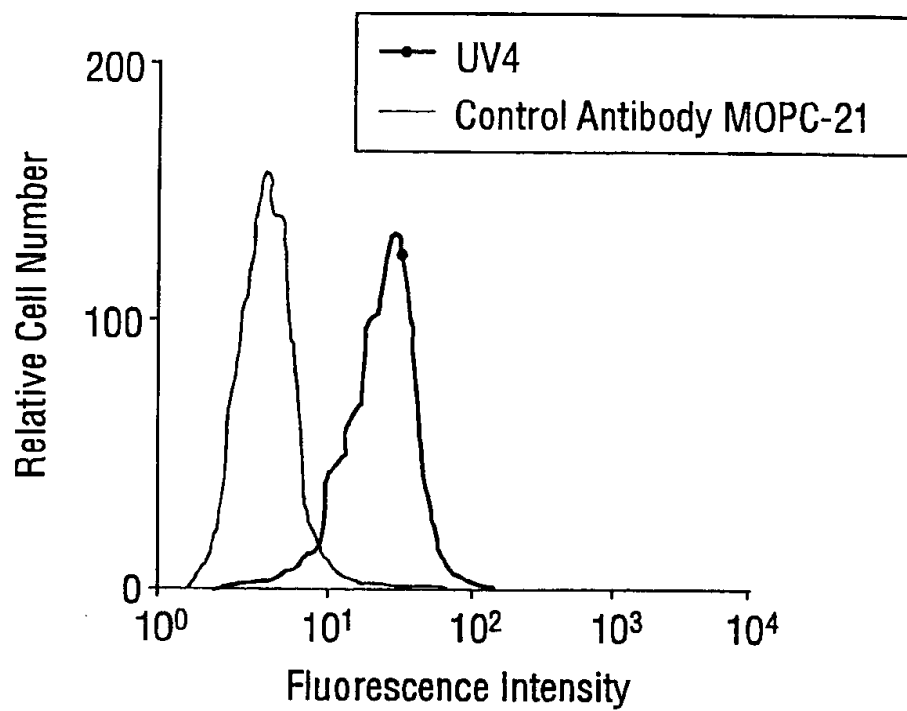


FIG. 3A

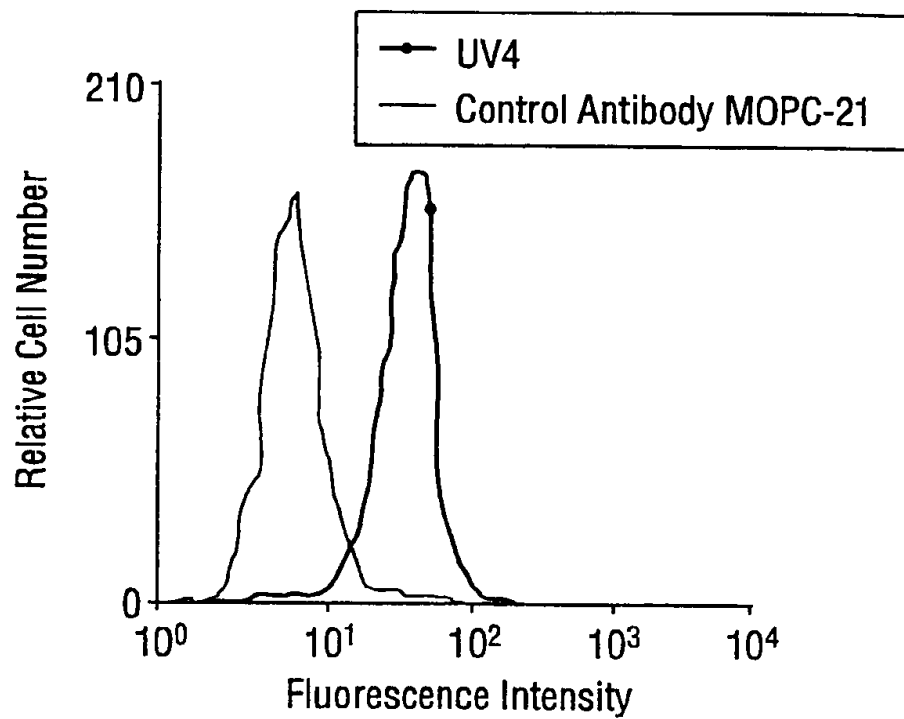


FIG. 3B



4/7

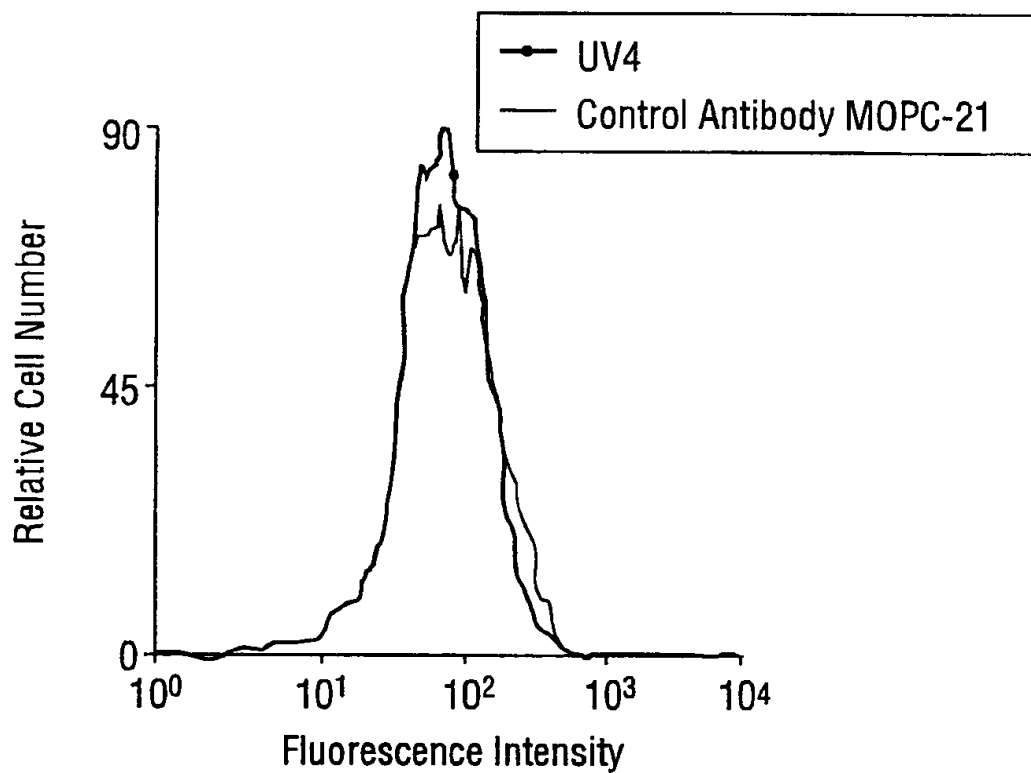


FIG. 3C

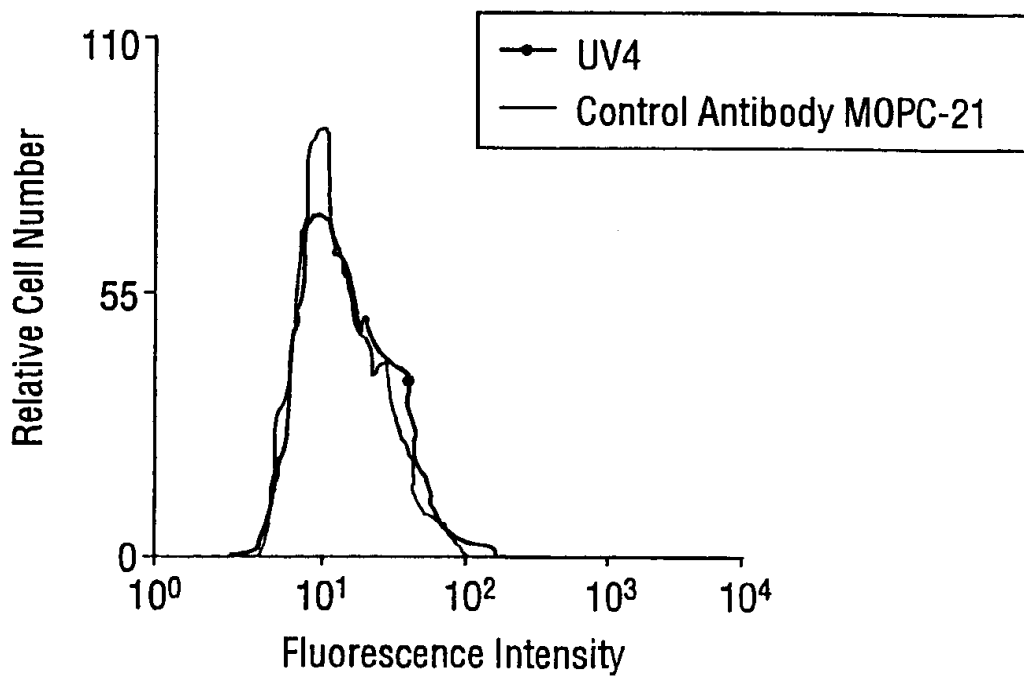


FIG. 3D

5/7

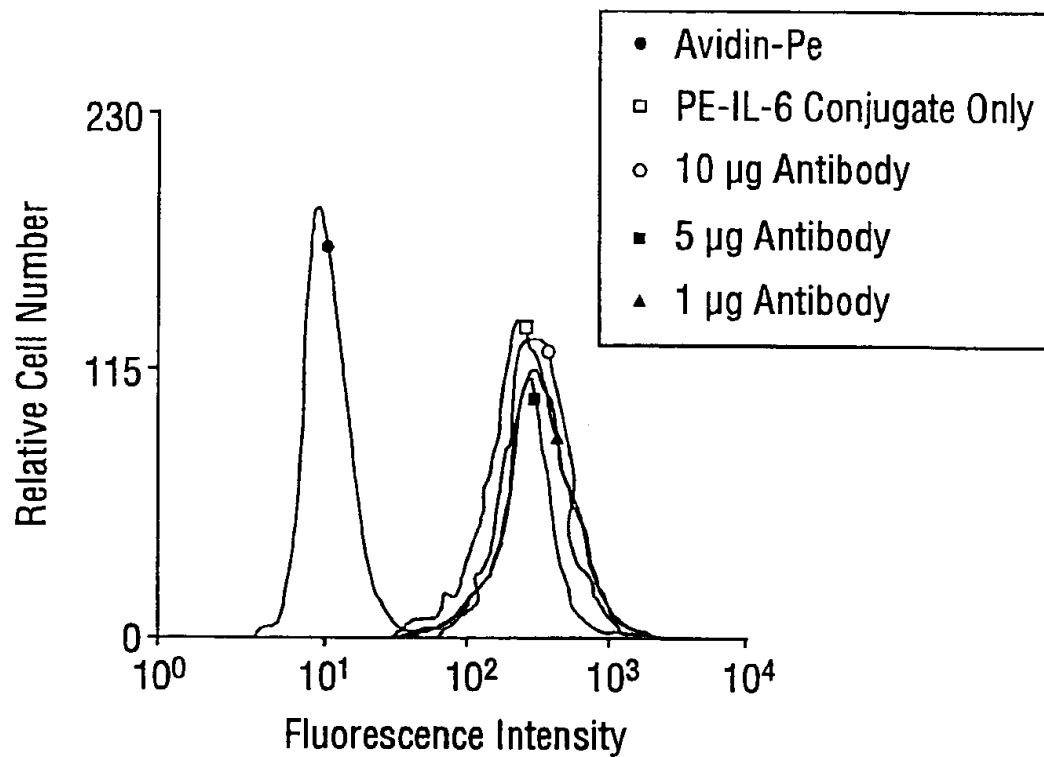


FIG. 4A

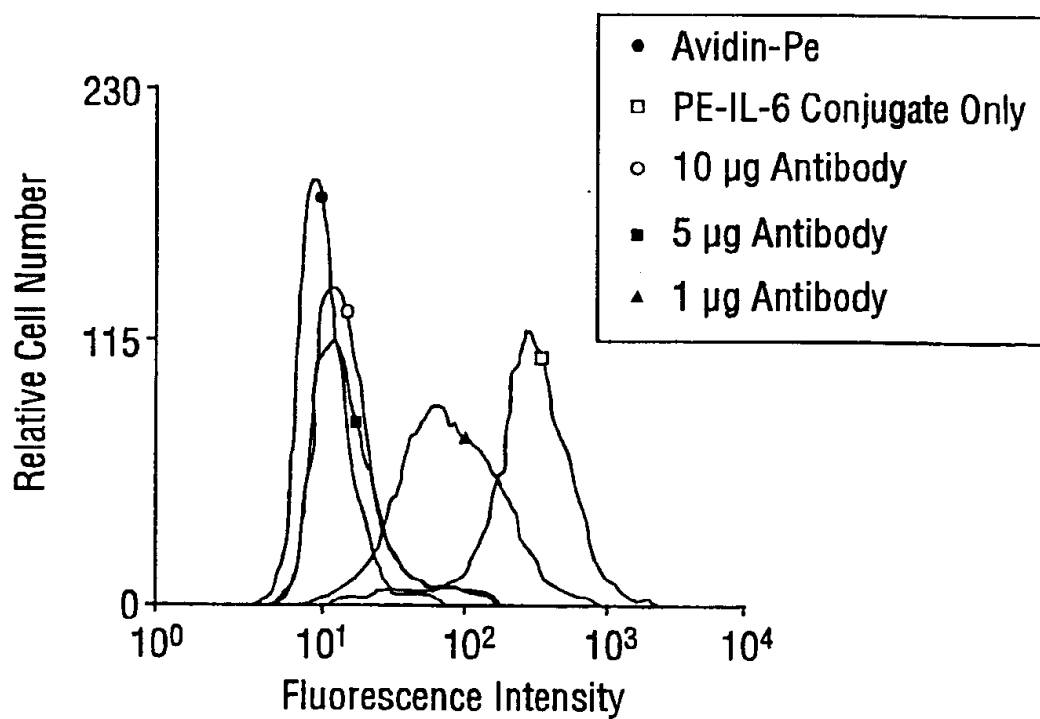


FIG. 4B

6/7

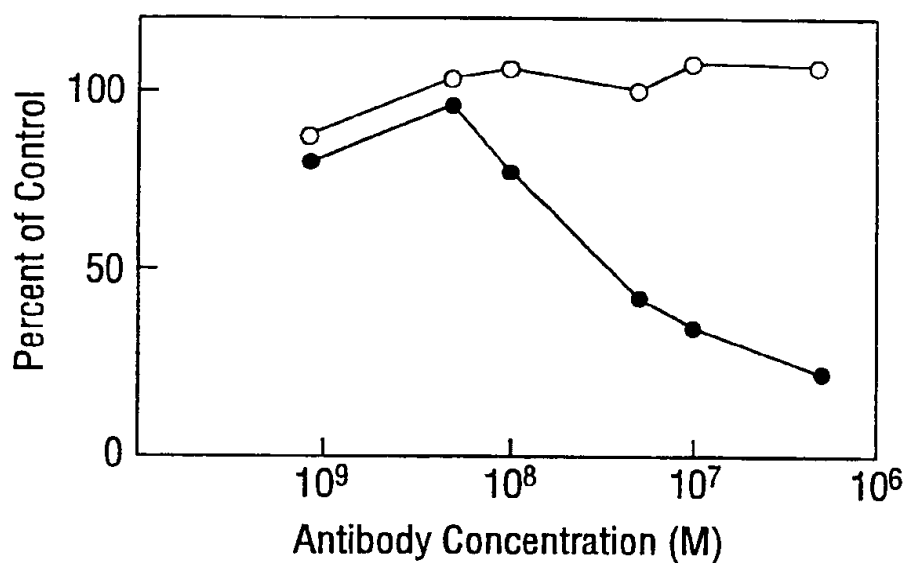


FIG. 5A

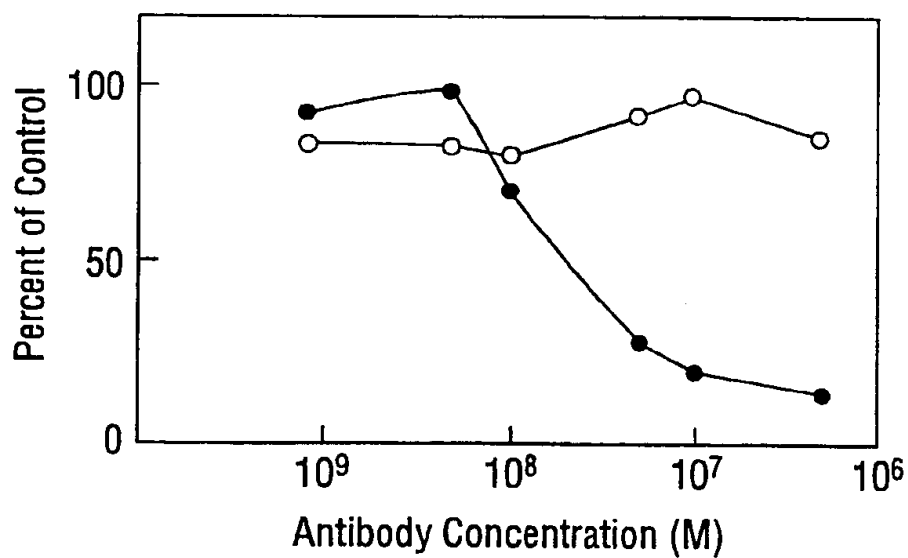


FIG. 5B

7/7

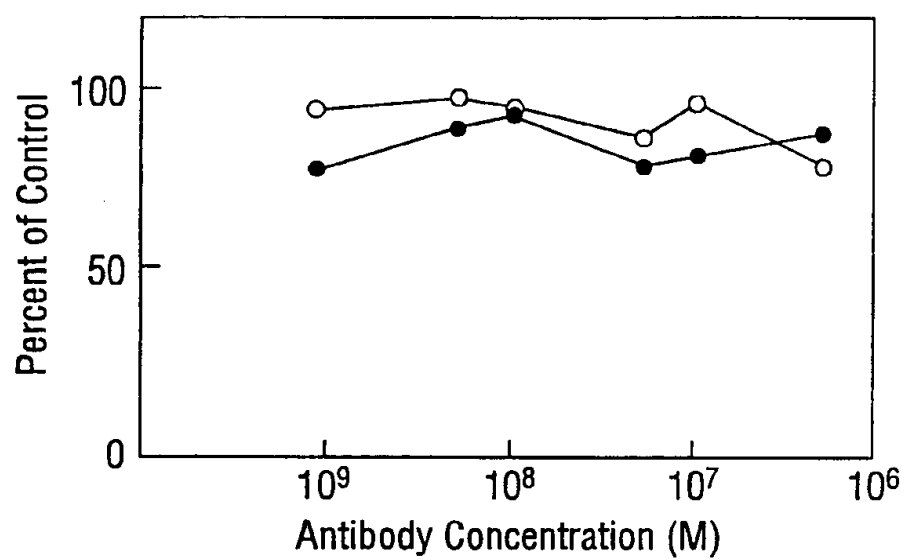


FIG. 5C

## INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 94/11305

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/28 C12N5/20 A61K39/395 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| X          | HYBRIDOMA,<br>vol.12, no.5, October 1993, NEW YORK NY,<br>USA<br>pages 621 - 630<br>Y. HUANG ET AL. 'A monoclonal anti-human<br>IL-6 receptor antibody inhibits the<br>proliferation of human myeloma cells.'<br>see the whole document<br>----         | 1-10                  |
| A          | CANCER RESEARCH,<br>vol.53, no.4, 15 February 1993,<br>PHILADELPHIA PA, USA<br>pages 851 - 856<br>K. SATO ET AL. 'Reshaping a human antibody<br>to inhibit the interleukin 6-dependent<br>tumor cell growth.'<br>see the whole document<br>-----<br>-/- | 1-10                  |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

23 January 1995

Date of mailing of the international search report

06 -02- 1995

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Nooij, F

## INTERNATIONAL SEARCH REPORT

Intern: al Application No

PCT/US 94/11305

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|--|--|-----------------------|
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| A  | HYBRIDOMA,<br>vol.10, no.1, February 1991, NEW YORK NY,<br>USA<br>pages 137 - 146<br>D. NOVICK ET AL. 'Monoclonal antibodies to<br>the soluble human IL-6 receptor: affinity,<br>purification, ELISA, and inhibition of<br>ligand binding.'<br>see the whole document<br>---                                   | 1-10                  |
| A  | THE JOURNAL OF IMMUNOLOGY,<br>vol.143, no.9, 1 November 1989, BALTIMORE<br>MD, USA<br>pages 2900 - 2906<br>Y. HIRATA ET AL. 'Characterization of IL-6<br>receptor expression by monoclonal and<br>polyclonal antibodies.'<br>see the whole document<br>---   | 1-10                  |
| A  | EP,A,0 409 607 (T. KISHIMOTO) 23 January<br>1991<br>see the whole document<br>---  | 1-10                  |
| A  | JOURNAL OF IMMUNOLOGICAL METHODS,<br>vol.163, no.2, 9 August 1993, AMSTERDAM,<br>THE NETHERLANDS<br>pages 217 - 223<br>T. SAITO ET AL. 'Preparation of monoclonal<br>antibodies against the IL-6 signal<br>transducer, gp130, that can inhibit<br>IL-6-mediated functions.'<br>see the whole document<br>----- | 1-10                  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/11305

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
REMARK : Although claims 4-8 (partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Information on patent family members

PCT/US 94/11305

Form PCT/ISA/210 (patent family annex) (July 1992)